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13. ABSTRACT (Maximum 200 Words) The estrogen receptor α (ER α) plays an important role in breast cancer and a large fraction of ER α positive breast cancers respond to tamoxifen. We cloned a novel p21 activated kinase, termed PAK6, which binds to the androgen receptor and the 4-hydroxytamoxifen liganded ER α . PAKs are a family of serine/threonine kinases that bind to and are regulated by the Rho family small GTPases, Cdc42 and Rac. We found that PAK6 transcripts were expressed in normal mammary epithelium and a possible alternative splice product in breast cancer cell lines, indicating that PAK6 may play a role in breast cancer. The purpose of this research was to assess PAK6 expression in breast cancer and to determine whether it contributes to ER α function in breast cancer cells or to tamoxifen responses. The studies have established that PAK6 is expressed in normal breast epithelium and breast cancer, that it contributes to the growth of breast cancer cells, and that increased PAK6 can antagonize the growth suppressing effects of tamoxifen. The reagents generated during the course of this study are available and can be used to further explore the molecular mechanisms by which PAK6 contributes to the growth of breast cancer cells.			
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INTRODUCTION

The classical estrogen receptor, estrogen receptor α (ER α) plays an important role in breast cancer development and a large fraction of ER α positive breast cancers respond to treatment with tamoxifen. We cloned a novel p21 activated kinase (PAK), termed PAK6, which binds to the androgen receptor (AR) and selectively to the 4-hydroxytamoxifen (OHT) liganded ER α . PAKs are a family of serine/threonine kinases that bind to and are regulated by the active (GTP bound) form of the Rho family small (p21) GTPases, Cdc42 and Rac. We found that PAK6 transcripts were expressed in normal mammary epithelium and our preliminary data showed a possible alternative splice product in breast cancer cell lines, indicating that PAK6 may play a role in breast cancer. The purpose of this research was to assess PAK6 expression in breast cancer and to determine whether it contributes to ER α function in breast cancer cells or to tamoxifen responses.

BODY

The proposal consisted of three tasks, which are outlined below. Progress towards completion of each task over the course of the funding period is then summarized.

Task 1. Determine the structure, cellular distribution, and activation state of endogenous PAK6 protein in normal breast and breast cancer cell lines

- a. Develop PAK6 antibodies (months 1-12)
- b. Assess endogenous PAK6 using these antibodies (months 6-24)
- c. Clone and sequence endogenous PAK6 from breast cancer cell lines (months 1-12)

Task 2. Determine whether PAK6 contributes to ER α function and cell growth in breast cancer cell lines

- a. Develop anti-sense methods to downregulate PAK6 (months 6-24)
- b. Develop dominant negative PAK6 constructs (months 1-18)
- c. Assess the functional consequences of blocking PAK6 (months 12-36)

Task 3. Determine whether PAK6 contributes to tamoxifen sensitivity in breast cancer

- a. Determine whether tamoxifen blocks PAK6 activation (months 18-30)
- b. Determine whether the tamoxifen-ER α -PAK6 complex mediates cell cycle arrest (months 12-24)

Develop PAK6 antibodies (Task 1a). A rabbit polyclonal antibody was generated against PAK6 by immunizing with GST-PAK6 fusion protein. The GST-PAK6 fusion protein was then coupled covalently to agarose beads and used to affinity purify the antibody. The specificity of the antibody is shown in figure 1. The PAK6 antibody was tested and titered by immunoblotting against lysates from CV1 and MCF7 cells transfected with HA epitope tagged PAK6 (tag at the N-terminus). The anti-PAK6 antibody used at approximately 250 ng/ml (1:100 dilution) readily detects a 75-80 kDa band that is identical to the band recognized by a monoclonal anti-HA antibody (Fig. 1). The anti-PAK6 also detects a second band at 65 kDa that is not present in untransfected cells. This is a proteolytic product with the N-terminal removed, as it is not seen with the HA tag antibody. A doublet at about 50 kDa is non-specific as it is recognized by the anti-rabbit secondary antibody (and to a lesser extent by the anti-mouse secondary) in MCF7 cells, but not other cell types. No further bands were detected, supporting the specificity of the PAK6 Ab.

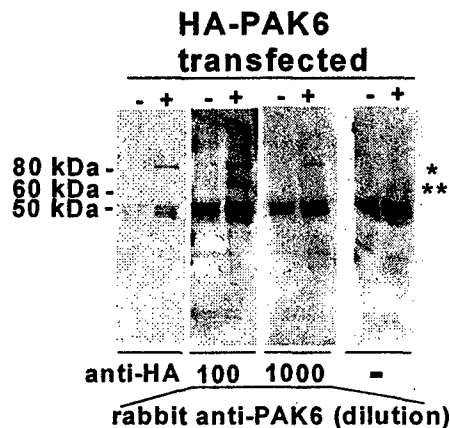


Figure 1. Specificity and titration of affinity purified anti-PAK6 antibody. Lysates from MCF7 cells transfected with HA tagged PAK6 are shown in (+) lanes and the untransfected in (-) lanes. The first panel is immunoblotted using mouse monoclonal anti-HA antibody. The second and third are 1:100 (250 ng/ml) and 1:1000 (25 ng/ml) dilutions of affinity purified anti-PAK6 antibody. The fourth is without primary antibody. Single or double asterixes indicate the intact PAK6 (*) and a proteolytic product (**). The strong doublet at about 50 kDa is a protein in MCF7 that reacts with the secondary antibodies, which is not seen in most other cells examined.

Assess endogenous PAK6 using these antibodies (Task 1b). The affinity purified PAK6 antibodies have been used to assess endogenous PAK6 protein expression in breast cancer cells, as well as in prostate cancer cells. Proteins were extracted from a series of cell lines and immunoblotted with the PAK6 versus control nonimmune antibody. Screens of breast cancer cell lines have revealed detectable PAK6 in most cells, with highest levels in MDA-231 (Fig. 2A). The size of the protein is consistent with the predicted size of endogenous PAK6 (75 kDa), and a lower band is the proteolytic product observed above with transfected PAK6. PAK6 was also expressed in each of the prostate cancer cell lines examined (LAPC4, LNCaP, CWR22Rv1, and PC3) (Fig. 2B).

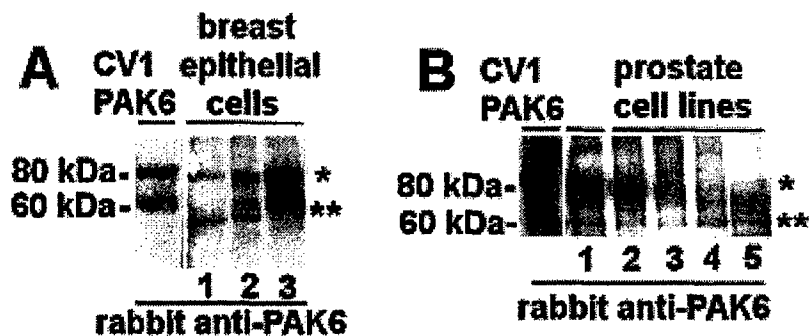


Figure 2. Immunoblot analysis for PAK6 expression in breast and prostate cancer cell lines. Equal amounts of protein (50 ug) from the indicated tumor cell lines, or from PAK6 transfected CV1 cells, were immunoblotted with anti-PAK6 (1:100 dilution). A, breast epithelial cells, Lane 1-HS578t, Lane 2-MDA-231, Lane3-normal breast epithelial cells. B, prostate cancer cells, Lane1-MCF7 (breast), Lane2-LAPC4, Lane3-LNCaP, Lane4-CWR22Rv1, Lane5-PC3.

Immunohistochemistry and immunofluorescence were used to assess the cellular distribution of PAK6. Both transfected PAK6 (not shown) and endogenous PAK6 in MCF7 cells was localized primarily to the cell membrane (Fig. 3). This localization was consistent with a role in cell signaling or regulating cell motility.



Figure 3. Cellular localization of PAK6. MCF7 breast cancer cells cultured on coverslips were immunostained with affinity purified anti-PAK6 antibody. No staining was observed with control nonimmune antibody (not shown).

Immunohistochemistry was used to assess PAK6 expression in clinical breast cancer samples. As shown in figure 4A, PAK6 was expressed in non-neoplastic mammary epithelium. The immunostaining was on the plasma membrane and cytoplasmic (Fig. 4A). Immunostaining of breast cancer samples also showed PAK6 expression (Fig. 4B). The staining was variable, but was not markedly increased and was generally comparable to normal epithelium. These results confirmed that PAK6 was expressed *in vivo* in normal breast epithelium and in breast cancer.

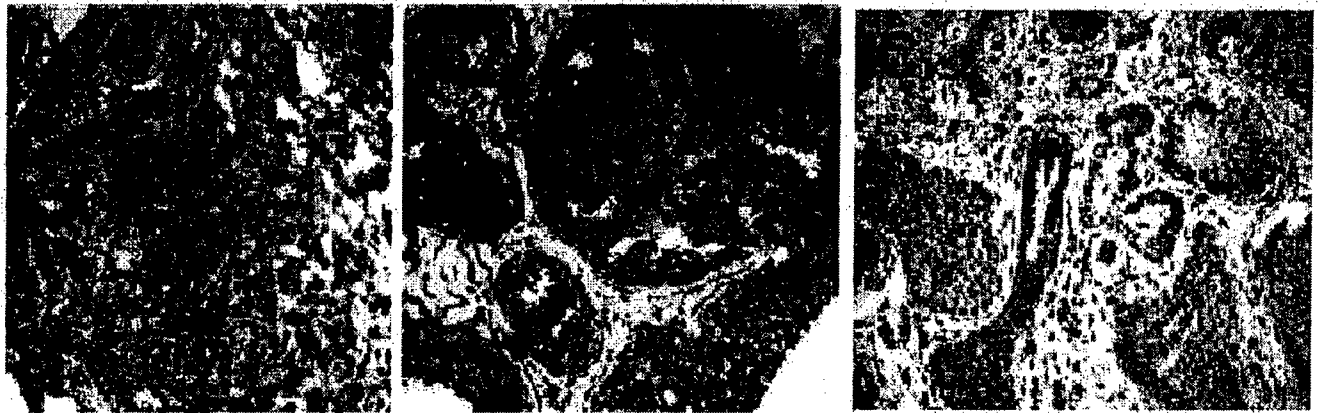


Figure 4. PAK6 expression in breast and breast cancer. Tissues were immunostained with anti-PAK6 (A and B), or nonimmune control antibody (C). A, non-neoplastic breast; B and C, breast cancer.

PAK6 was also expressed in normal prostate epithelium and in prostate cancer (Fig. 5A and B). Significantly, increased PAK6 expression was observed in a subset of metastatic prostate cancers (Fig. 5C). Based on these results, we plan further follow-up immunohistochemical studies of advanced metastatic breast cancers as well as prostate cancers.

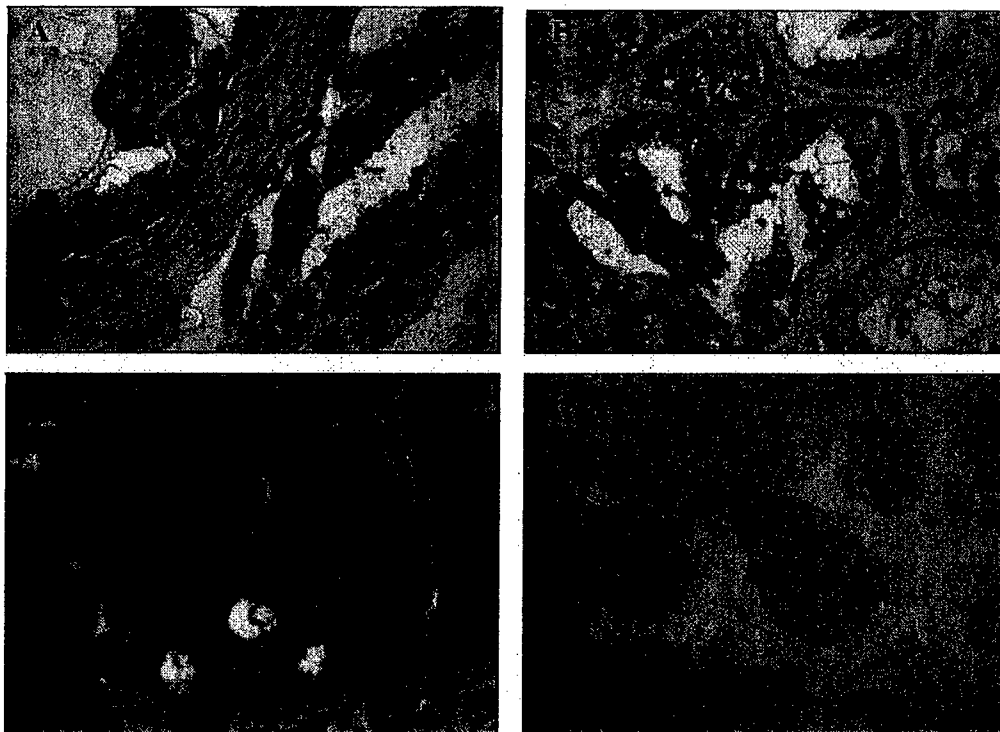


Figure 5. PAK6 expression in prostate and prostate cancer. Tissues sections were immunostained with affinity purified anti-PAK6 antibody (A-C), or nonimmune rabbit antibody (D), followed by biotin-anti-rabbit and AP-Streptavidin. A, non-neoplastic prostate epithelium; B, primary prostate cancer; C, metastatic prostate cancer; D, negative control.

Clone and sequence endogenous PAK6 from breast cancer cell lines (Task 1c). Our preliminary studies had suggested an alternative splice product for PAK6 in breast cancer cells. To identify this potential alternative transcript, we carried out a series of 5' and 3' RACE (Rapid Amplification of cDNA Ends) cloning experiments using breast cancer derived RNA samples. These experiments employed multiple primers in the 5' and 3' ends, but did not identify an alternative splice product. As showed above, our protein expression data similarly failed to reveal multiple isoforms. Based on these results, we conclude that the PAK6 expressed by breast cancer cells is identical to the molecule we cloned initially from prostate cancer.

Develop anti-sense methods to downregulate PAK6 (Task 2a). In order to assess the biological functions of endogenous PAK6 expressed by breast cancer cell lines, we proposed to develop methods to downregulate PAK6 expression. Therefore, we have generated small interfering RNA oligomers (siRNA) and tested their ability to decrease expression of endogenous PAK6. A pool of PAK6 siRNAs was first tested by transfection into MDA231 cells, followed by immunoblotting for PAK6. As shown in figure 6, these siRNA can markedly suppress the expression of endogenous PAK6. We have also produced plasmids that express this siRNA and are now deriving cell lines with markedly decreased PAK6 expression. These lines are currently being derived and will be made available for further functional studies.

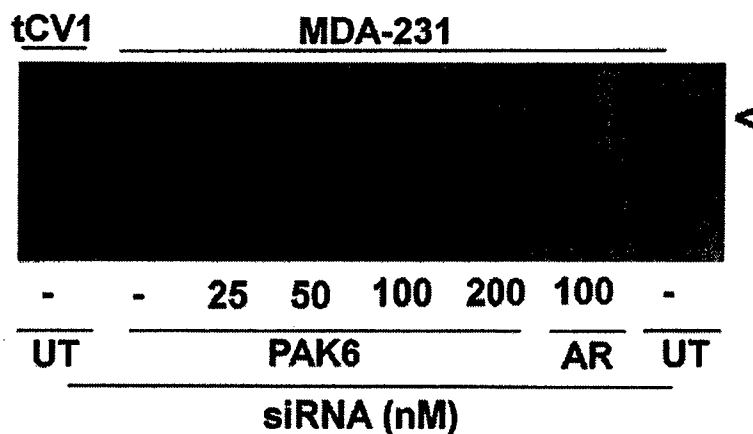


Figure 6. Downregulation of endogenous PAK6 by siRNA. MDA-231 cells were transfected using Lipofectamine for 24 hours with the indicated concentrations of siRNA for PAK6 or AR control. Cells were harvested after 3 more days and lysates were immunoblotted with anti-PAK6 antibody. UT, untransfected controls. The first lane is CV1 cells transfected with HA epitope tagged PAK6, which runs slightly higher.

Develop dominant negative PAK6 constructs (Task 2b). A mutant that can function as a dominant negative PAK6 was generated by mutating a critical lysine in the kinase domain to methionine (K to M). As shown in figure 7, this mutation completely abrogates PAK6 kinase activity. By site directed mutagenesis we also generated a PAK6 mutant that is constitutively active (serine to asparagines, SN, Fig. 7). Additional mutants that abrogate Cdc42 binding have also been generated (not shown). Transfectants expressing these mutant proteins have been generated and are now available for further functional study.

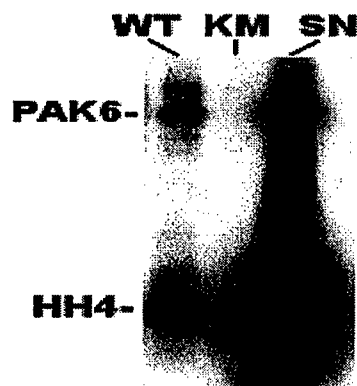


Figure 7. Dominant negative kinase dead PAK6 mutant. CV1 cells were transfected with PAK6 wildtype (WT), kinase dead lysine to methionine (KM), or constitutively active serine to asparagines (SN) mutants. The proteins were immunoprecipitated and kinase assays were carried out using histone H4 (HH4) as a substrate. The position of autophosphorylated PAK6 is also shown.

Assess the functional consequences of blocking PAK6 (Task 2c). To determine whether PAK6 plays a role in regulating tumor cell growth, we examined the effect of knocking down endogenous PAK6 in MDA-231 breast cancer cells. Cells were transfected with PAK6 specific or negative control (AR) siRNA and cell growth was assessed using MTS assays. As shown in figure 8, the PAK6 siRNA specifically suppressed the growth of MDA-231, indicated a role for PAK6 in stimulating cell growth.

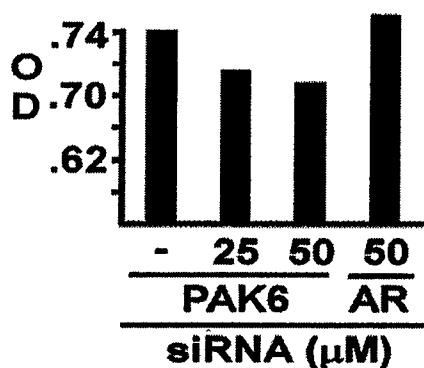


Figure 8. Suppression of cell growth by PAK6 siRNA. MDA-231 breast cancer cells expressing endogenous PAK6 were transfected with siRNA for PAK6 or androgen receptor (AR, which is not expressed in these cells). Cell growth was then measured by MTS assay after 5 days. Similar results were observed over 4-7 days (not shown).

Determine whether tamoxifen blocks PAK6 activation (Task 3a). One key to understanding how PAK6 contributes to the biology of breast cancer cells is to determine how its kinase activity is regulated. Based on our published data showing that PAK6 can be activated by androgen receptor, we proposed to examine PAK6 activation by the estradiol or tamoxifen liganded ER α . These studies showed that PAK6 activity was not modulated by ER α (data not shown). Therefore, we next attempted to identify other mechanisms that regulate PAK6 kinase activity. These studies have shown that PAK6 activity is dually regulated by MKK6 and p38 MAP kinase, and that the PAK family is unique in having its kinase domain regulated both by serine/threonine and tyrosine phosphorylation. These studies, which are detailed in the attached manuscript by Kaur et al. (submitted for publication to J. Biol. Chem.), demonstrate a novel link between MAP kinase and PAK6 activation.

Determine whether the tamoxifen-ER α -PAK6 complex mediates cell cycle arrest (Task 3b). Due to the interaction between PAK6 and the tamoxifen liganded ER α , we hypothesized that PAK6 may modulate breast cancer responses to tamoxifen. To test this hypothesis, we generated stable transfectants of the MCF7 cell line expressing increased levels of PAK6 (MCF7-PAK6 cells). The effect of hydroxytamoxifen (the active metabolite of tamoxifen) on the growth of these cells was then determined. As shown in figure 9, the growth suppressing effects of hydroxytamoxifen were substantially reduced in the cells with increased PAK6. In conjunction with the above data, these results indicate that PAK6 sequestration by the tamoxifen liganded ER α contributes to the growth suppressive effects of this drug.

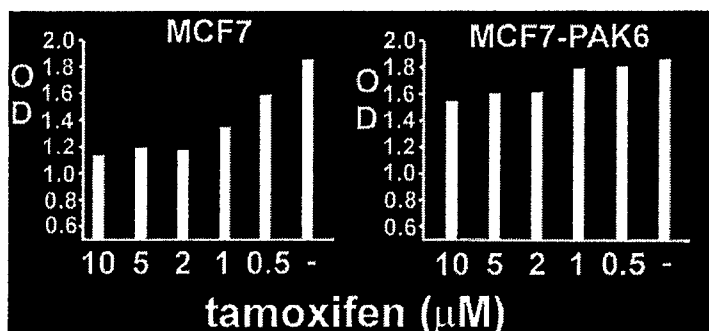


Figure 9. PAK6 expression decreases growth suppression by tamoxifen. MCF7 or a stable PAK6 expressing line of MCF7 were treated with hydroxytamoxifen at the indicated concentrations and cell growth was assessed by MTS assay at day 5.

KEY RESEARCH ACCOMPLISHMENTS

- PAK6 cloning
- demonstration of PAK6 interaction with ER α and AR
- generation of PAK6 antibodies
- affinity purification of PAK6 antibodies
- demonstration of PAK6 protein in breast and prostate cancer cells
- demonstration of PAK6 in normal and neoplastic breast epithelium
- generation of PAK6 mutants
- generation of MCF7 cell lines expressing increased levels of wild type and mutant PAK6
- generation of siRNA to downregulate PAK6 expression
- demonstration that PAK6 is activated by MKK6 and p38 MAP kinase

REPORTABLE OUTCOMES

- manuscript: Lee et al., 2002 (appended)
- manuscript: Kaur et al., submitted for publication (appended)
- immunohistochemical data on PAK6 expression in breast and breast cancer (manuscript in preparation)
- functional consequences of suppressing and increasing PAK6 expression in breast cancer cells (manuscript in preparation)

CONCLUSIONS

The tasks outlined in the proposal have been largely completed. Importantly, these studies have established that PAK6 is expressed in normal breast epithelium and breast cancer, that it contributes to the growth of breast cancer cells, and that increased PAK6 can antagonize the growth suppressing effects of tamoxifen. Moreover, the reagents generated during the course of this study are available and can be used to further explore the molecular mechanisms by which PAK6 contributes to the growth of breast cancer cells.

REFERENCES

Lee SR, Ramos SM, Ko A, Masiello D, Swanson KD, Lu ML, and Balk SP. 2002. AR and ER Interaction with a p21-Activated Kinase (PAK6). *Mol Endo* 16:85-99

APPENDICES

manuscript : Kaur et al., submitted

**Activation of p21 Activated Kinase 6 (PAK6) by MAP Kinase Kinase 6 and p38
MAP Kinase**

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Running title: PAK6 activation via P38 MAP kinase/MKK6 pathway

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Summary

The p21 activated kinases (PAKs)¹ contain an N-terminal Cdc42/Rac interactive binding (CRIB) domain, which in the group 1 PAKs (PAK1, 2, and 3) regulates the activity of an adjacent conserved autoinhibitory domain. In contrast, the group 2 PAKs (PAK4, 5, and 6) lack this autoinhibitory domain and are not activated by Cdc42/Rac binding, and the mechanisms that regulate their kinase activity have been unclear. This study found that basal PAK6 kinase activity was repressed by a p38 MAP kinase antagonist and could be strongly stimulated by a constitutively active MAP kinase kinase 6 (MKK6), an upstream activator of p38 MAP kinases. Mutation of a consensus p38 MAP kinase target site at serine 165 decreased PAK6 kinase activity. Moreover, PAK6 was directly activated by MKK6, and mutation of tyrosine 566 in a consensus MKK6 site (threonine-proline-tyrosine, TPY) in the activation loop of the PAK6 kinase domain prevented activation by MKK6. PAK6 activation by MKK6 was also blocked by mutation of an autophosphorylated serine (serine 560) in the PAK6 activation loop, indicating that phosphorylation of this site is necessary for MKK6-mediated activation. PAK4 and PAK5 were similarly activated by MKK6, consistent with a conserved TPY motif in their activation domains. The activation of PAK6 by both p38 MAP kinase and MKK6 suggests that PAK6 (and likely other PAKs) have a unique and specialized role in the cellular response to stress.

Introduction

P21-activated kinases (PAKs) were originally identified as serine/threonine protein kinases that bound to and were activated by the p21 GTPases, GTP-Cdc42 and -Rac. Binding of p21 is mediated by an N-terminal Cdc42/Rac interactive binding (CRIB) domain, and biochemical and crystal structure analyses of PAK1 have shown that the CRIB domain regulates the inhibitory activity of an adjacent autoinhibitory domain (AID). In the absence of p21 GTPase binding, PAK1 exists as an autoinhibited dimer in which the N-terminal AID of one PAK1 molecule in the dimer binds to the other catalytic domain and blocks its function. Binding of GTP-Cdc42 or -Rac causes the AID to dissociate from the catalytic domain and activates its kinase activity, with subsequent phosphorylation of sites in the N-terminal regulatory domain and in the activation loop of the kinase domain serving to maintain the activated state (1,2).

The N-terminal CRIB domain and AID are highly conserved in human PAK2 and PAK3, and these PAKs have been categorized with PAK1 as group 1 PAKs. PAK6 was initially identified in yeast two-hybrid screens for androgen receptor interacting proteins (3,4). PAK6 has a C-terminal kinase domain with homology to the group 1 PAKs and an N-terminal CRIB domain. However, PAK6 lacks the conserved AID and is not stimulated by ligation of its CRIB domain, which binds selectively to GTP-Cdc42 (3). Human PAK4 and PAK5 similarly lack the conserved AID, and along with PAK6 comprise the group 2 PAKs (5).

Group 1 PAKs (PAK1, PAK2, and PAK3) are involved in the regulation of diverse cellular processes such as cell motility, morphology, cytoskeletal reorganization, and gene regulation. Much less is known about the regulation and function of group 2 PAKs

(PAK4, PAK5, and PAK6). PAK4 is expressed ubiquitously, and activated PAK4 has been shown to mediate cytoskeleton reorganization and filopodia formation (6,7). Targeted disruption of PAK4 results in embryonic lethality. PAK5 is highly expressed in brain and neuronal tissues and has been shown to promote neuron outgrowth during development. RNA blot analyses have shown that PAK6 is expressed most highly in brain and testes, and at lower levels in multiple tissues including prostate and breast. In transfection studies, PAK6 has been shown to suppress androgen receptor transcriptional activity, and similarly bind to and repress estrogen receptor (3).

The mechanisms that regulate the group 2 PAKs are unclear, but the absence of a conserved AID indicates that the modes of regulation differ from the group I PAKs. In the current study, we describe a novel mechanism of PAK6 regulation by the MKK6-p38 MAP kinase pathway. Our results demonstrate that MKK6 activates PAK6 by targeting two separate sites, a consensus p38 MAP kinase substrate site (ser-165) and a tyrosine (tyr-566) in the activation loop of the kinase domain. Significantly, this tyrosine is part of an MKK6 substrate motif (threonine-x-tyrosine) that is conserved in the group 1 and 2 PAKs, but is otherwise largely restricted to activation loops of MAP kinases, where it undergoes direct dual phosphorylation by MAP kinase kinases. This study further shows that MKK6-mediated activation does not alter the autophosphorylation of a regulatory serine in the activation loop of PAK6 (ser-560), which is also conserved in the activation loop of all PAKs. Moreover, this serine is required for MKK6-p38 MAP kinase activation of PAK6. Taken together, the results in this study indicate that PAK6 is regulated by MKK6 and p38 MAP kinase, and that the PAK6 activation loop is regulated by both MKK6 and autophosphorylation. This conservation of the activation loop in the

group 1 and group 2 PAKs suggest that all members may share this latter novel dual regulatory mechanism.

Experimental Procedures

Materials- Reagents: p38 MAP kinase inhibitor SB203508 and MEK1 inhibitor PD98059 were purchased from LC Laboratories (Woburn, MA). PKA activator forskolin and histone H4 were purchased from Sigma (St. Louis, MO). JNK inhibitor SP600125, PI3K inhibitor LY 294002, and MEK1 inhibitor U1026 were purchased from Calbiochem (San Diego, CA). Protein-A-conjugated Sepharose beads were from Amersham/Pharmacia. Monoclonal antibodies against P-Y and p38 MAP kinase were from Upstate Biotechnology (Lake Placid, NY) and monoclonal antibody 12CA5 against hemaagglutinin antigen (HA) from Berkeley Antibody (Berkeley, CA). Anti-serum against Phospho-PAK4(ser474)/PAK5(Ser602)/PAK6(Ser560) was purchased from Cell Signaling Technology (Burlington MA). Quick Change mutagenesis kit was from Stratagene Inc. (La Jolla, CA). Anti-PAK6 polyclonal anti-serum was generated against GST fused with PAK6 residues #115-#383.

Expression vectors and constructs: PAK6 was cloned in-frame with a N-terminal HA-tag containing pcDNA3.0 vector (Invitrogen). N-terminal tagged MKK6 (EE) was a gift from Dr. R. Davis (8). Generation of S165A and Y566F point mutations were done by PCR-based site-directed mutagenesis Quick-Change Kit following manufacturer's instructions. Mutation primers used for S165A are: CCG TGG CCC GAG CCA CAG GAA CCA CGG GTC CTG CCC AAT GGG and CCG TGG CCC GAG CCA CAG GAA CCA CGG GTC CTG CCC AAT GGG. Primers used for Y566F are: TCC CTG GTG GGA ACC CCC TTC TGG ATG GCT CCT GAA GTG and CAC TTC AGG AGC CAT CCA GAA GGG GGT TCC CAC CAG GGA. The site-directed mutagenesis was performed based on manufacturer's protocol.

Cell culture and transient transfection: HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM; high glucose), supplemented with antibiotics and 10% fetal bovine serum. Cells were transfected by electroporation with a total of 10 μ g of plasmid DNA using a BioRad Gene Pulser (BioRad, Hercules, CA).

Gel electrophoresis and immunoblotting: Proteins were separated by SDS-PAGE with a standard reducing protocol. Following electrophoresis, proteins were electroblotted to a nitrocellulose membrane. The protein bands were visualized by ponceau S red staining. Blots were blocked by 5% nonfat dry milk, 0.05% Tween 20, and 1% BSA in Tris-buffered saline (10 mM Tris (pH8.0), 135 mM NaCl). Immunoblotting was performed with designated antibodies and visualized with an enhanced chemiluminescence detection system (ECL, Pierce, Supersignal, Rockford, IL) following the manufacturer's protocol.

Immunoprecipitation: Immunoprecipitation of PAK6 and proteins containing phosphotyrosine was employed following a standard protocol. In brief, cells were lysed in immunoprecipitation RIPA buffer containing 50 mM Tris (pH 7.4), 135 mM NaCl, 1% V/V Triton X-100, 0.25% W/V deoxycholate, and 0.05% W/V SDS and supplemented with protease inhibitors (PMSF 2 mM, DIFP 5 mM, pepstatin 5 μ g/ml, EDTA 1 mM). Lysates were cleared by centrifugation at 12,000 x g for 30 min at 4°C. Supernatants were incubated with individual antibodies (1 μ g) and protein A sepharose beads (20 μ l packed beads) at 4°C for 1 h. At the end of incubation, beads were washed 5 times with lysis buffer. The resulting immunoprecipitated immunocomplexes were solubilized in 40 μ l of Laemmli sample buffer, resolved by SDS-PAGE, and transferred to a

nitrocellulose membrane. The protein complex was detected by western blot analysis and developed by ECL (Pierce, Supersignal).

In vitro kinase assay: Kinase reactions of immunoprecipitated PAK6 were performed in kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl₂, 2 mM MnCl₂ and 2 mM DTT, 200 μ M ATP) supplemented with 2.5 μ g/reaction of histone H4 and 20 μ Ci/reaction of radioactive ATP. The reactions were incubated for 30 min at 30°C and stopped by addition of sample buffer containing SDS. The reactions were resolved by SDS-PAGE, and autoradiograph of radio-labeled protein was performed.

Results

PAK6 is inhibited by p38 MAP kinase antagonist. In contrast to PAK1, PAK6 exhibits readily detectable basal kinase activity even in the absence of exogenous stimulation. To determine the molecular mechanisms that regulate PAK6 kinase activity, we tested a group of agents with known specificity either as inhibitors or as activators of their respective pathways. HEK293 cells were transiently transfected with HA-tagged PAK6 for 24 hours and then treated with drugs for 1 hour prior to immunoprecipitation with an anti-HA antibody. Kinase activities in the immunoprecipitates were then measured by an *in vitro* kinase assays, using histone H4 as an exogenous substrate. Among 9 tested agents, only the p38 MAP kinase inhibitor SB203580 exhibited inhibitory effects on PAK6 kinase activity, with reduced autophosphorylation and reduced phosphorylation of the exogenous histone H4 substrate (Fig. 1). Modest increases in autophosphorylation were observed with U1026, a MEK1 inhibitor, and with forskolin, a protein kinase A stimulator. However, these increases in autophosphorylation did not correlate with increased kinase activities toward the exogenous histone H4 substrate.

PAK6 is activated by p38 MAP kinase upstream activator MKK6. Inhibition of PAK6 kinase activity by SB203580 suggested that the p38 MAP kinase pathway was regulating PAK6 activity. To further test this possibility, we cotransfected HEK293 cells with HA-tagged PAK6 and a constitutively active MKK6, MKK6(EE), an upstream activator of p38 MAP kinase (9,10). The effect on PAK6 kinase activity was then assessed by an *in vitro* immunoprecipitation kinase assay. As shown in figure 2A,

MKK6(EE) caused an increase in PAK6 autophosphorylation and histone H4 phosphorylation. Immunoblotting of the immunoprecipitates with the anti-HA antibody confirmed that PAK6 protein expression was not altered, indicating that MKK6(EE) increased PAK6 kinase activity (Fig. 2B).

The involvement of p38 MAP kinase in this PAK6 activation by MKK6(EE) was examined by assessing the inhibitory effect of SB203580. Significantly, while SB203580 markedly downregulated PAK6 activity in the absence of MKK6(EE), it only partially inhibited the MKK6(EE)-induced activation of PAK6 (Fig. 2C). This partial inhibition was consistent with the high level of p38 MAP kinase activation in the MKK6(EE) transfected cells (Fig. 2D). However, the substantial PAK6 activation in the MKK6(EE) transfected and SB203580 treated cells also suggested a p38 MAP kinase independent mechanism for PAK6 activation.

To further address the role of p38 MAP kinase in PAK6 activation, we attempted to identify a site that was phosphorylated by p38 MAP kinase. Aided by an online kinase substrate site analysis, Scansite (<http://scansite.mit.edu>), we identified a potential p38 MAP kinase phosphorylation site at serine 165 of PAK6. To characterize the role of Ser 165 in p38 MAP kinase-mediated PAK6 activation, we generated a serine-to-alanine (S165A) substitution mutant of PAK6 by site-directed mutagenesis. Consistent with SB203580 downregulation of PAK6 kinase activity, substitution of Ser 165 with alanine dramatically reduced basal PAK6 kinase activity (Fig. 3A, lanes 1 versus 3) as well as MKK6-stimulated activity (Fig. 3A, lanes 2 versus 7). However, despite the downregulation of kinase activity in the PAK6 S165A mutant, it remained responsive to MKK6-induced activation in a dose-dependent manner (Fig. 3A). Figure 3B shows that

the serine-to-alanine mutation, or MKK6 cotransfection, did not markedly alter PAK6 protein expression. Taken together, these findings indicated another mechanism for PAK6 activation that was independent of p38 MAP kinase phosphorylation of serine 165.

PAK6 activity in response to MKK6 is regulated by sites in the kinase domain.

The result that the S165A PAK6 mutant remained responsive to MKK6 indicated that additional target site(s) in PAK6 might be involved in p38 MAP kinase-mediated activation. To test this hypothesis, a series of PAK6 deletion mutants were employed to map additional region(s) of PAK6 that might participate in the MKK6-p38 MAP kinase-induced activation. Although the basal activities varied, cotransfected MKK6(EF) remained effective in upregulating the kinase activities of PAK6 deletion mutants that extend from the N-terminal to the region covering only the catalytic domain (Δ 368 deletion mutant) (Fig. 4A). The levels of expression of wild-type and deletion mutants in the control and MKK6(EF) co-transfected groups were similar, as shown in figure 4B. This result suggested that additional sites in the kinase domain were susceptible to activation by MKK6(EF).

The PAK6 catalytic domain contains a serine in the activation loop at position 560 that is conserved in PAK4 and PAK5. A recent study using a phospho-specific antibody indicated that this serine 560 in PAK6 was phosphorylated in unstimulated cells, although its mechanism of phosphorylation and role in regulating kinase activity have not been determined (11). This serine residue corresponds to threonine 423 in the activation loop of PAK1, 2, and 3, and mutation of this threonine to glutamic acid

(T423E) in PAK1 to mimic a negatively charged phospho-threonine results in constitutive activation (12,13). In contrast to this result with PAK1, substitution of serine 560 in PAK6 with a negatively charged residue (glutamic or aspartic acid) failed to enhance kinase activity (Fig. 5A). The marked decrease in phosphorylation of PAK6 versus the histone substrate is consistent with serine 560 being an autophosphorylation site that contributes to PAK6 kinase activity. Comparable levels of expression of the wild-type and PAK6 mutants are shown in figure 5B.

The involvement of serine 560 phosphorylation in response to MKK6-p38 MAP kinase-induced PAK6 activation was evaluated by immunoblotting with an anti-phospho-PAK6(Ser 560) antibody (11). As shown in figure 5C (lanes 1 and 2), the level of PAK6 ser 560 phosphorylation was not altered by MKK6(EE) cotransfection as compared to that of the wild-type kinase. As expected, the S560A, S560D, and S560E mutants of PAK6 did not exhibit immunoreactivity with anti-Ser 560 antibody (Figure 5C, lanes 3 to 5). Finally, although serine 560 was not a direct target of MKK6-p38 MAP kinase, we addressed whether it was necessary for PAK6 activation by this pathway. Significantly, the PAK6 S560A and S560E mutants could not be activated by transfection with MKK6(EE) (Fig. 5D). Taken together, these results indicate that serine 560 is necessary for PAK6 activation by MKK6, but that the activation is not mediated by phosphorylation of this site.

PAK6 is phosphorylated on tyrosine upon MKK6 activation. The PAK6 activation loop also contains a threonine-proline-tyrosine (TPY) sequence (residues 564-566) that resembles the substrate motif (TxY) recognized by MKK6 (14,15). This motif is present

in the activation loop of MAP kinases and PAK1-6, but not in most other kinases (see Fig. 9A). MKK6 is a dual-specific kinase that recognizes and phosphorylates both threonine and tyrosine residues on the TxY motif of its substrate. The identification of TPY within the PAK6 activation loop suggested that PAK6 might be a direct substrate of MKK6. If this is the case, then one should detect increased tyrosine phosphorylation of PAK6 upon MKK6(EE)-induced activation. Reciprocal immunoprecipitation/western blot (IP-western) analyses were performed to test this possibility.

PAK6 was immunoprecipitated from transfected cells with a polyclonal antibody (B16) raised against PAK6 and then immunoblotted with anti-phosphotyrosine antibody (4G10). An increase in tyrosine phosphorylation in the anti-PAK6 immunoprecipitates was detected when PAK6 was co-expressed with MKK6(EE) (Fig. 6A). In contrast, no change in tyrosine phosphorylation of PAK6 was detected when it was cotransfected with the constitutively active MKK1(DD), a related member of the MAP kinase kinase family (16). Immunoblotting with anti-HA showed that the immunoprecipitates contained comparable levels of total PAK6 (Fig 6B). In the reciprocal experiment, lysates were immunoprecipitated with the anti-phosphotyrosine 4G10 antibody and then immunoblotted for PAK6. As shown in figure 6C, PAK6 was immunoprecipitated specifically in cells cotransfected with MKK6.

To further address the phosphorylation of Tyr 566 by MKK6, a dual-specificity phosphatase, MKP-1 (mitogen kinase phosphatase-1), which can dephosphorylate both threonine and tyrosine on the TxY motif (17), was tested in IP-western experiments. MKP-1 cotransfection in the absence of MKK6(EE) markedly decreased the basal tyrosine phosphorylation of PAK6 (Fig. 7A and B, lanes 1 versus 3). MKP-1

cotransfection similarly reduced the level of MKK6-induced tyrosine phosphorylation of PAK6 (Fig. 7A and B, lanes 2 versus 4). Figure 7C demonstrates that total PAK6 expression levels were not decreased by MKP-1 (lanes 1 versus 3, and 2 versus 4). Finally, *in vitro* kinase assays were carried out to assess the correlation between loss of tyrosine phosphorylation induced by MKP-1 and PAK6 kinase activity. Consistent with the tyrosine phosphorylation results, MKP-1 downregulated basal and MKK6-stimulated PAK6 autophosphorylation and kinase activity on the exogenous histone H4 substrate (Figure 7D).

Substitution of Tyr 566 with phenylalanine downregulates PAK6 activation by MKK6. To assess more directly the involvement of Tyr 566 phosphorylation in PAK6 activation, a mutant PAK6 (Y566F) was generated by substituting the Tyr 566 residue with a phenylalanine. This mutation markedly reduced the level of basal PAK6 tyrosine phosphorylation (Fig. 8A and B, lanes 1 versus 3). Similarly, the Y566F mutation markedly reduced the tyrosine phosphorylation stimulated by MKK6 (Fig. 8A and B, lanes 2 versus 4). Figure 8C demonstrates that the wild-type and mutant PAK6 constructs were expressed at comparable levels. These results indicate that Tyr 566 is a major site of basal and MKK6-stimulated tyrosine phosphorylation, although this may not be the only site, as the Y566F mutation did not completely eliminate MKK6-induced tyrosine phosphorylation.

We next examined the effects of the Y566F mutation on basal and MKK6-stimulated PAK6 kinase activity. Substitution of Tyr 566 with phenylalanine diminished basal PAK6 autophosphorylation and kinase activity towards the exogenous histone H4

substrate (Fig. 8D, lanes 1 versus 3). Significantly, the Y566F mutation abrogated MKK6-stimulated PAK6 kinase activity (Fig. 8D, lanes 2 versus 4). Therefore, although MKK6 may directly or indirectly phosphorylate additional tyrosines, this site appears to be most critical for regulating kinase activity. Taken together, these data suggest that MKK6 activates PAK6 by direct phosphorylation of the TxY motif located within the activation loop.

Regulation of other PAKs by the MKK6/p38 MAP kinase pathway. As indicated above, the activation segments of PAK1-6 all contain similarly positioned TPY sequences, located four amino acids carboxy to their autophosphorylated regulatory serines (PAK4-6) or threonines (PAK1-3) (Fig. 9A). This TxY motif is absent from most other serine/threonine kinases, and the positioning of the motif in the MAP kinases is distinct. These observations suggest that activation induced by MKK6 may be common amongst PAK family kinases. Therefore, additional members of the PAKs (HA-tagged PAK1, PAK4 and PAK5) were tested in the same cotransfection studies coupled with *in vitro* kinase assays.

Among these kinases, PAK5 strongly responded to MKK6(EF) co-transfection with increased kinase activity in a fashion similar to PAK6 (Fig. 9B). PAK4 had less basal kinase activity, but this was also stimulated by MKK6(EF). In contrast, PAK1 was inactive in both the absence and presence of MKK6(EF), consistent with the strong CRIB domain-regulated autoinhibitory peptide in the PAK1 N-terminus. Immunoblotting with an anti-HA antibody confirmed that each of the kinases was expressed at comparable levels (Fig. 9C). These results indicate that members of the group 2 PAK

family (PAK4, 5, and 6) share a common mechanism of being stimulated via the MKK6-p38 MAP kinase pathway. In contrast, the group 1 PAKs may not be stimulated by MKK6/p38 MAP kinase or may require an initial Rac/Cdc42-mediated activation step.

Discussion

PAK6 is classified as a PAK family member based on homology in the kinase domain and its N-terminal CRIB domain. However, in contrast to PAK1 and the other group 1 PAKs, PAK6 kinase activity is not stimulated by Cdc42 or Rac binding, and the mechanisms that regulate its kinase activity have been unclear. This study found that basal PAK6 kinase activity was repressed by a p38 MAP kinase antagonist and could be strongly stimulated by activation of the MKK6-p38 MAP kinase pathway. A role for p38 MAP kinase in directly regulating PAK6 was further supported by a marked decrease in kinase activity upon mutation of a consensus target site at serine 165. PAK6 was also directly activated by MKK6, and this activation was dependent upon Tyr 566 being present in the activation loop of the PAK6 kinase domain. Significantly, this tyrosine is part of an MKK6 dual-specific kinase substrate motif, TxY, that is found in the activation loop of MAP kinases and in the other PAKs, but is absent from most other kinases. These results and further findings detailed in this study indicate that PAK6 kinase activity is uniquely regulated by both autophosphorylation and MAP kinase-mediated phosphorylation of residues in its activation loop. Moreover, the highly conserved activation loops in PAK1-6, each containing the TxY motif, indicate that this dual regulatory mechanism may be common to the PAK family.

The kinase activity of PAK1 is regulated by an autoinhibitory domain (AID) in the N-terminal half of the molecule downstream of the CRIB domain, which binds to and represses the catalytic domain (1,2,12,18-21). The binding of GTP-Rac or GTP-Cdc42 to the CRIB domain causes the AID to dissociate from the catalytic domain, with subsequent phosphorylation of residues in the AID and of Thr-423 in the activation loop

of the catalytic domain (12,13,18,22). In contrast to PAK1 and the other group 1 PAKs (PAK2 and PAK3), this AID is not conserved in PAK6 or in the other group 2 PAKs (PAK4 and PAK5), and the kinase activities of the group 2 PAKs are not stimulated by Cdc42 binding. Nonetheless, the group 2 PAKs contain serines at a position homologous to the autophosphorylated threonine-423 of PAK1 (serine 560 in PAK6), and this serine appears to be constitutively phosphorylated in PAK6.

Thr-423 in PAK1 and ser-560 in PAK6 are located at the center of the activation loop within the catalytic domain. A threonine or serine at this position is conserved among ser/thr kinases and in many cases is autophosphorylated upon activation (5). Crystallographic studies of PAK1 indicate the phosphorylation of this residue stabilizes the interaction between the activation loop and substrate (1). Consequently, substituting Thr-423 with a negatively charged glutamic acid residue renders PAK1 constitutively active. In PAK4, mutating the corresponding Ser-473 to glutamic acid also results in constitutive kinase activity (23,24). Phosphorylation of PAK6 at Ser-560 was recently demonstrated using a phospho-specific anti-PAK6-Ser560 antibody (11). However, we found that substituting the PAK6 Ser-560 with glutamic acid or aspartic acid did not enhance kinase activity, unlike a similar substitution in PAK1 or PAK4. Nonetheless, an S560A mutant had reduced kinase activity and, significantly, was not further stimulated by MKK6. This result indicates that phosphorylation of Ser-560 may be required to prime PAK6 for activation by MKK6.

Although PAK6 does not appear to have an AID, and ser-560 phosphorylation appears to be constitutive, we cannot yet rule out the possibility ser-560 phosphorylation negatively regulates PAK6 interaction with a undefined inhibitory

domain. One recent report describes as the "autoinhibitory" domain of the PAK5 a region that bears almost no sequence homology to other members of the PAK family (25). This region (residues 60-180) is located directly downstream of the PAK5 CRIB domain and appears to be able to downregulate PAK5 kinase activity *in vitro*. This result raises the interesting possibility that regions immediately following the CRIB domain of group 2 family PAKs may regulate the catalytic domain. If this is the case for PAK6, then the identification of serine-165 as a p38 MAP kinase substrate site would suggest that PAK6 activation may be initiated by phosphorylation of this site, and that this activation signal may then be amplified by ser-560 phosphorylation and subsequent MKK6 phosphorylation of the activation loop.

The TxY substate motif for MKK6 is conserved in the same position in all of the PAK family members. Moreover, the kinase activities of PAK4 and PAK5 were also stimulated by MKK6, consistent with the group 2 PAKs being regulated by MKK6. In contrast, PAK1 was not activated by MKK6, suggesting it may not be MKK6-regulated. However, this may instead reflect the strong AID in PAK1 and the necessity to reverse this repression via Cdc42/Rac binding before phosphorylation of thr-423. Further studies are under way to determine whether PAK1 can be further activated by MKK6 subsequent to initial activation by GTP-Cdc42 or Rac.

Although the PAK and MAP kinase families appear to share a functional TxY MAP kinase kinase motif, its position is shifted towards the C-terminal of the activation loop in the PAKs. Moreover, PAKs differ from the MAP kinases in that they have a regulatory serine or threonine located in the center of the activation loop. Further structural studies are needed to determine precisely how phosphorylation at these

multiple sites affects the activation loop and kinase activity. Nonetheless, the shared mechanism of activation by PAK6 and p38 MAP kinase suggests that PAK6 (and likely other PAKs) have a unique and specialized role in the cellular response to stress-related signals.

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¹ Abbreviations: MKK6, MAP kinase kinase 6; PAK, p21 activated kinase; CRIB, Cdc42/Rac interactive binding domain; AID, auto-inhibitory domain; MKP-1, mitogen kinase phosphatase-1; WT, wild type; HA, hemagglutinin; MAP, mitogen-activated protein

Figure Legends:

Figure 1: Inhibition of PAK6 kinase activity by p38 MAP kinase inhibitor

SB203580. Various agents were used to treat HEK293 cells transiently transfected with HA-Pak6 for 24 h. Cells were treated with various pharmacological agents for 1 hr before being subjected to the immunoprecipitation protocol. PAK6 was immunoprecipitated from cell lysates with anti-HA MAb 12CA5 and its kinase activity assayed in the presence of Histone H4 and [$\gamma^{32}\text{P}$] ATP. Both auto-phosphorylation and substrate phosphorylation were analyzed after SDS-PAGE and autoradiography. The upper panel shows the auto-phosphorylation of Pak6. The lower panel shows the phosphorylation of exogenously added Histone H4 substrate. Dosage used: SB203580, 25 μM ; PD98059, 100 μM ; SP600125, 10 μM ; LY294002, 20 μM ; U1026, 50 μM ; and Forskolin, 10 μM .

Figure 2: MKK6-induced PAK6 activation is downregulated by SB203580. (A) 293 cells were transiently co-transfected with both HA-PAK6 and various dosages of constitutively active mutant MKK6 (EE). In vitro kinase activity was assessed by a kinase assay on the immunoprecipitated PAK6. A steady increase of PAK6 kinase activity in response to MKK6 (EE) dosage is clearly visible. (B) An equal amount of PAK6 from the immunoprecipitation was shown. (C) The effect of SB203580 on MKK6-activated PAK6 kinase activity was measured by an IP/kinase assay using cell lysates of 293 cells transiently co-transfected with MKK6 and PAK6 that were treated with or without SB203580. SB203580 partially inhibits the MKK6-induced PAK6 activation (lanes 3 and 4). (D) The phosphorylation status of p38 MAP kinase in response MKK6

activation was confirmed by western blot analysis using an anti-p38 phospho-specific antibody.

Figure 3: Substituting serine 165 with alanine downregulates MKK6-induced PAK6 activation. (A) 293 cells were transiently co-transfected with HA-PAK6 wt or S165A and with various dosages of constitutively active mutant MKK6(EE). In vitro kinase activities were determined by a kinase assay on the immunoprecipitated PAK6. Mkk6-induced activation is apparent in wt PAK6 (lanes 1 and 2). The PAK6 S165A mutant exhibits low basal kinase activities as compared to its wt counterpart (see lanes 1 and 3). A dose-dependent activation of mutated PAK6 kinase is demonstrated when S165A is co-transfected with an increasing dosage of MKK6. Panel (B) shows western blot of PAK6 wt and S165A mutants with equal amount of protein in the IP-kinase assay.

Figure 4: Mapping PAK6 domain susceptible to MKK6-induced activation. (A) 293 cells were co-transfected with MKK6 (EE) and HA-tagged PAK6 wt full-length (FL) and N-terminal deletion mutants $\Delta 190$, $\Delta 292$ or $\Delta 368$ respectively for 24 h. The kinase activity of PAK6 wt and N-terminal deletion mutants were determined by an IP-kinase assay using anti-HA 12CA5 MAb. The resulting The kinase activity was analyzed by SDS-PAGE and autoradiography. (B) Western blot analysis shows the equal levels of expression of PAK6 FL and deletions.

Figure 5: PAK6 does not respond to MKK6-induced activation with increased Ser560 phosphorylation. (A) 293 cells transiently co-transfected with HA-tagged PAK6 Ser560 substitution mutants, serine to alanine (S560A), serine to aspartic acid (S560D), or serine to glutamic acid (S560E), were immunoprecipitated by anti-HA 12CA5 monoclonal antibody and subjected in vitro kinase assay in the presence of human Histone H4 as exogeneous substrate. Wt PAK6 co-transfected with MKK6 serves as a positive control for PAK6 activation. (B) As expected, these mutants exhibit no immuno-reactivity toward the anti-ser560 phospho-specific antibody. (C) Western blot shows comparable amount of PAK6 protein in the immunoprecipitates. (D) PAK6 Ser560 substitution mutants, S560A and S560E, are not activated by co-transfected MKK6(EE).

Figure 6: Tyrosine phosphorylation of PAK6 by MKK6 but not by MKK1. 293 cells were co-transfected with HA-PAK6 and MKK6(EE) or MKK1(DD) and further incubated for 24 h. PAK6 was immunoprecipitated with anti-HA 12CA5 MAb or with anti-PY 4G10 MAb. The immunoprecipitates were resolved by SDS-PAGE followed by reciprocal western blot analysis. In both (A) and (C), an increased tyrosine phosphorylation of PAK6 is evident in MKK6(EE) co-transfected group. (B) Comparable levels of PAK6 in the anti-PAK6(B16) immunoprecipitates are demonstrated by western blot using anti-HA MAb.

Figure 7: Downregulation of MKK6-induced PAK6 tyrosine phosphorylation and kinase activation by dual-specificity phosphatase MKP-1. 293 cells were co-

transfected with HA-PAK6 and MKK6(EE) or MKP-1 and further incubated for 24 h. PAK6 was immunoprecipitated with anti-HA 12CA5 MAb or with anti-PY 4G10 MAb. The immunoprecipitates were resolved by SDS-PAGE followed by reciprocal western blot analysis. The results are shown in (A) and (B): MKP-1 de-phosphorylates PAK6 tyrosine residue under basal and MKK6 stimulated conditions, as evident from the reduced immunoreactivity of PAK6 with anti-PY 4G10 MAb. (C) Comparable amounts of PAK6 in lysates derived from each group as demonstrated by Western blot analysis. (D) Downregulation of PAK6 kinase activity by MKP-1 co-transfection is demonstrated by an IP-kinase assay using 293 cells co-transfected with PAK6 and with MKK6 (EE) or MKP-1. Kinase activity was determined by an in vitro kinase assay with [32 P]ATP using the anti-HA immunoprecipitated kinase and analyzed by SDS-PAGE followed by autoradiography.

Figure 8: Substitution of Tyr-566 with phenylalanine (Y566F) dampens MKK6-stimulated PAK6 activation. Levels of tyrosine phosphorylation were evaluated between PAK6 wt and Y566F mutant by IP/western blot analysis using 293 cells transiently expressing MKK6(EE) and PAK6 (wt or Y566F). PAK6 was immunoprecipitated with anti-HA 12CA5 MAb or with anti-PY 4G10 MAb. The immunoprecipitates were resolved by SDS-PAGE followed by reciprocal western blot. The results are shown in (A) and (B): reduced levels of tyrosine phosphorylation of PAK6 in basal and MKK6-stimulated conditions are evident in the Y566F group. (C) Western blot demonstrates the comparable amount of PAK6 in anti-HA immunoprecipitates. (D) In vitro IP-Kinase assay demonstrates downregulation of PAK6

kinase activity by substituting tyrosine-566 with phenylalanine (Y566F) at both basal non-stimulated and MKK6-stimulated conditions

Figure 9: Specificity of MKK6-induced PAK activation. (A) Sequence alignment of the activation loop region between catalytic subdomains VII and VIII of PAKs and MAP kinase family members. In both group 1 and group 2 PAKs, in addition to the previously defined autophosphorylated threonine or serin residues, a conserved TxY motif is also found within the activation loop. (B) Kinase activity of MKK6-modulated PAKs: 293 cells were co-transfected with MKK6(EE) and one of the following HA-tagged PAK family members: PAK1 , PAK4, PAK5, or PAK6. The kinase activity was determined by an in vitro kinase assay with anti-HA MAb immunoprecipitated kinases and analyzed by SDS-PAGE followed by autoradiography. (C) To determine the even level of expression in control and testing groups, half of the cell lysates from each transfection group were used for a western blot analysis with anti-HA MAb.

Figure 1: Inhibition of PAK6 kinase activity by p38 MAP kinase inhibitor SB203580

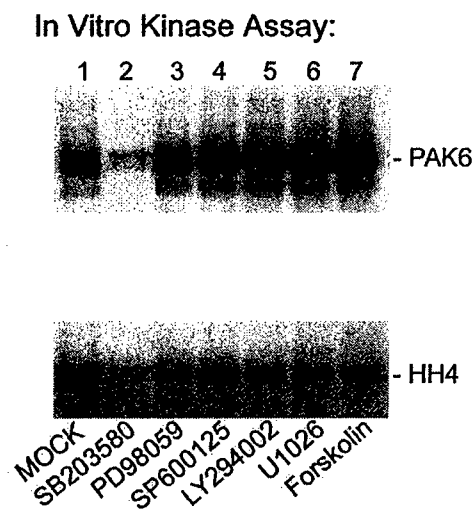


Figure 2: MKK6-induced PAK6 activation is downregulated by SB203580

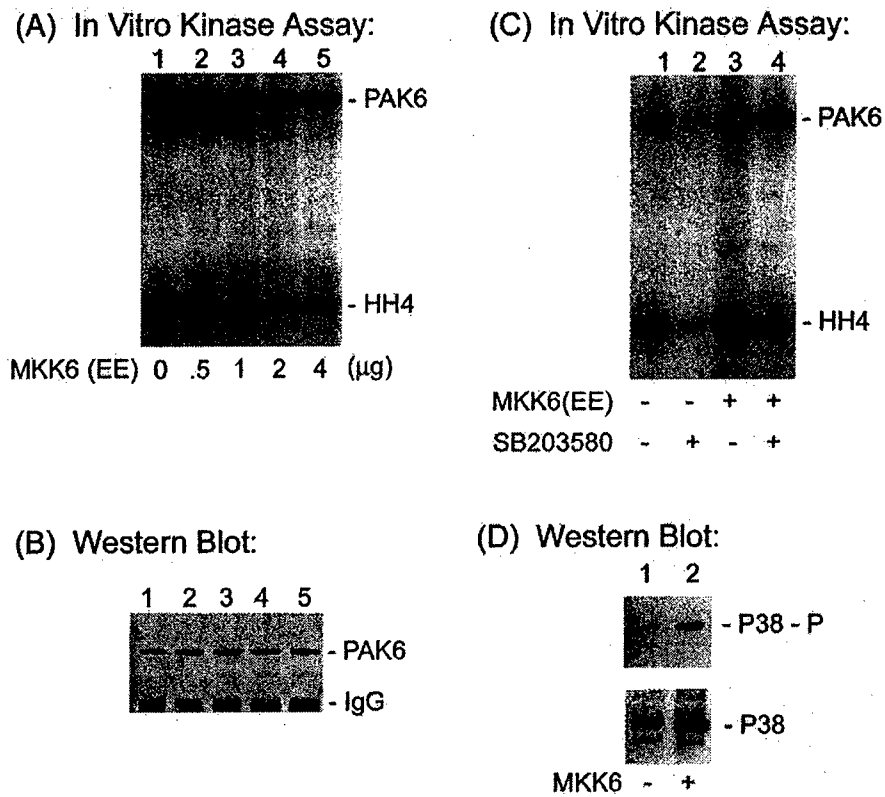
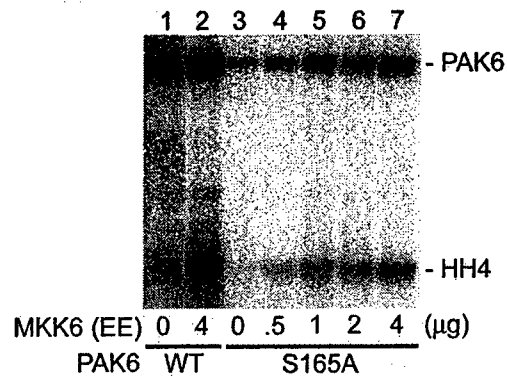


Figure 3: Substituting serine 165 with alanine downregulates MKK6-induced PAK6 activation

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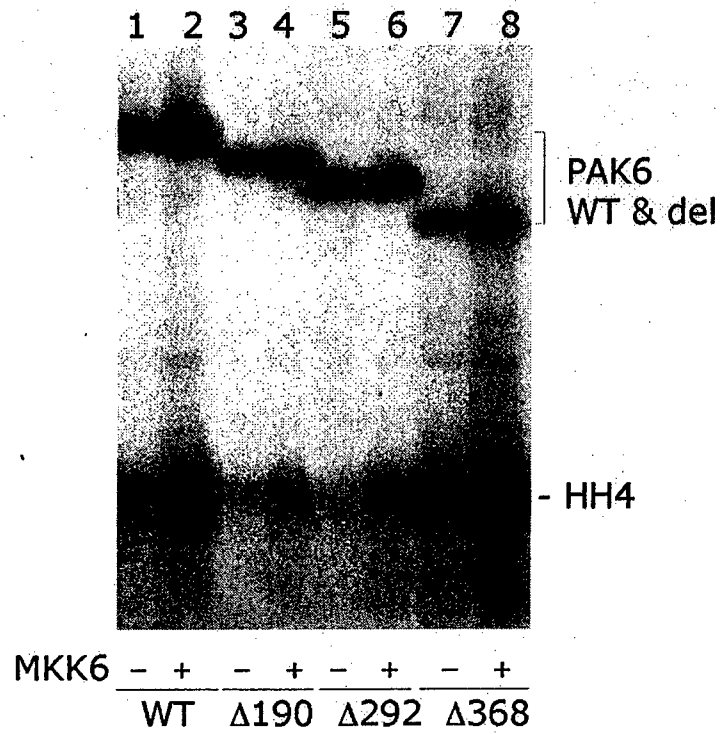


(B) Western Blot:



Figure 4: Mapping PAK6 domain susceptible to MKK6-induced activation

(A) In vitro Kinase Assay



(B) Western Blot

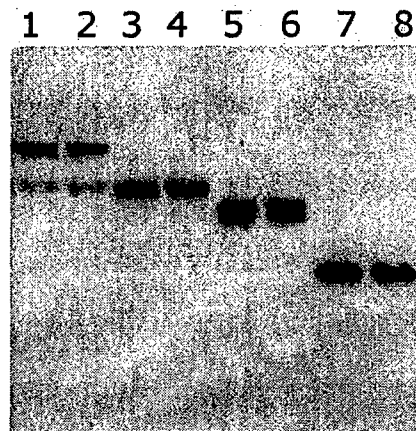
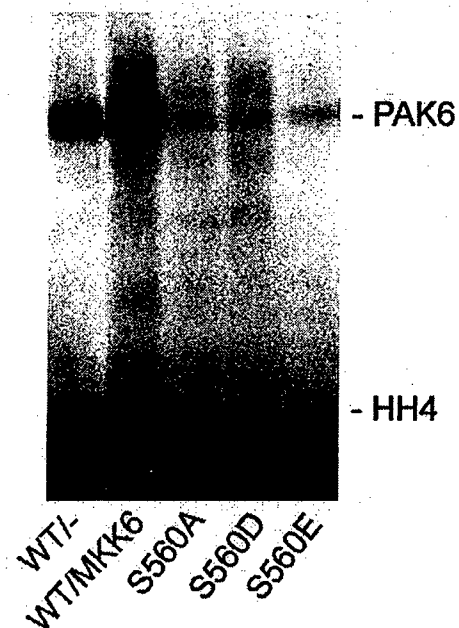
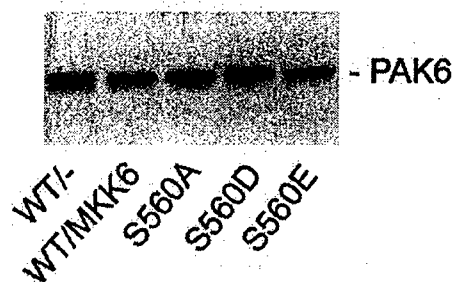


Figure 5: PAK6 does not respond to MKK6-induced activation with increased Ser560 phosphorylation.

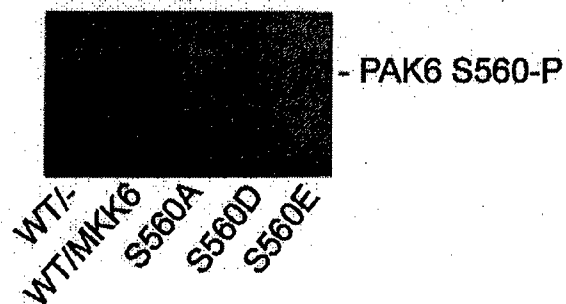
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(B) Western Blot:



(C) IP/Western Blot:



(D) In Vitro Kinase Assay:

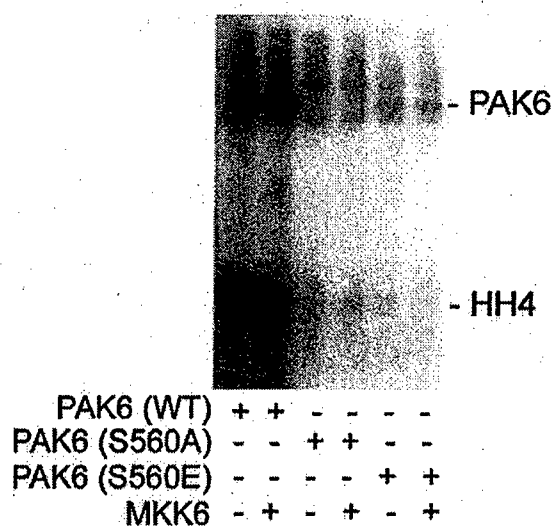
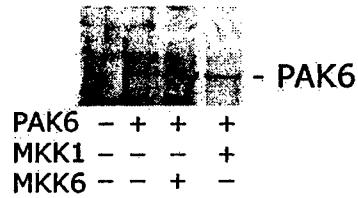
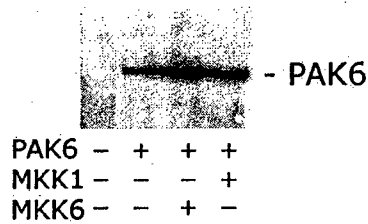


Figure 6: Tyrosine phosphorylation of PAK6 by MKK6 but not by MKK1

(A) Imppt: α -PAK6 (B16)
Blot: α -PY (4G10)



(B) Imppt: α -PAK6 (B16)
Blot: α -HA (12CA5)



(C) Imppt: α -PY (4G10)
Blot: α -PAK6

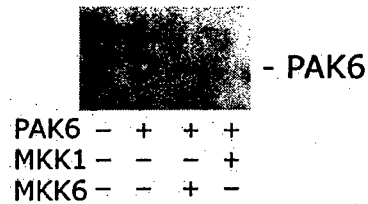


Figure 7: Downregulation of MKK6-induced PAK6 tyrosine phosphorylation and kinase activation by dual-specificity phosphatase MKP-1

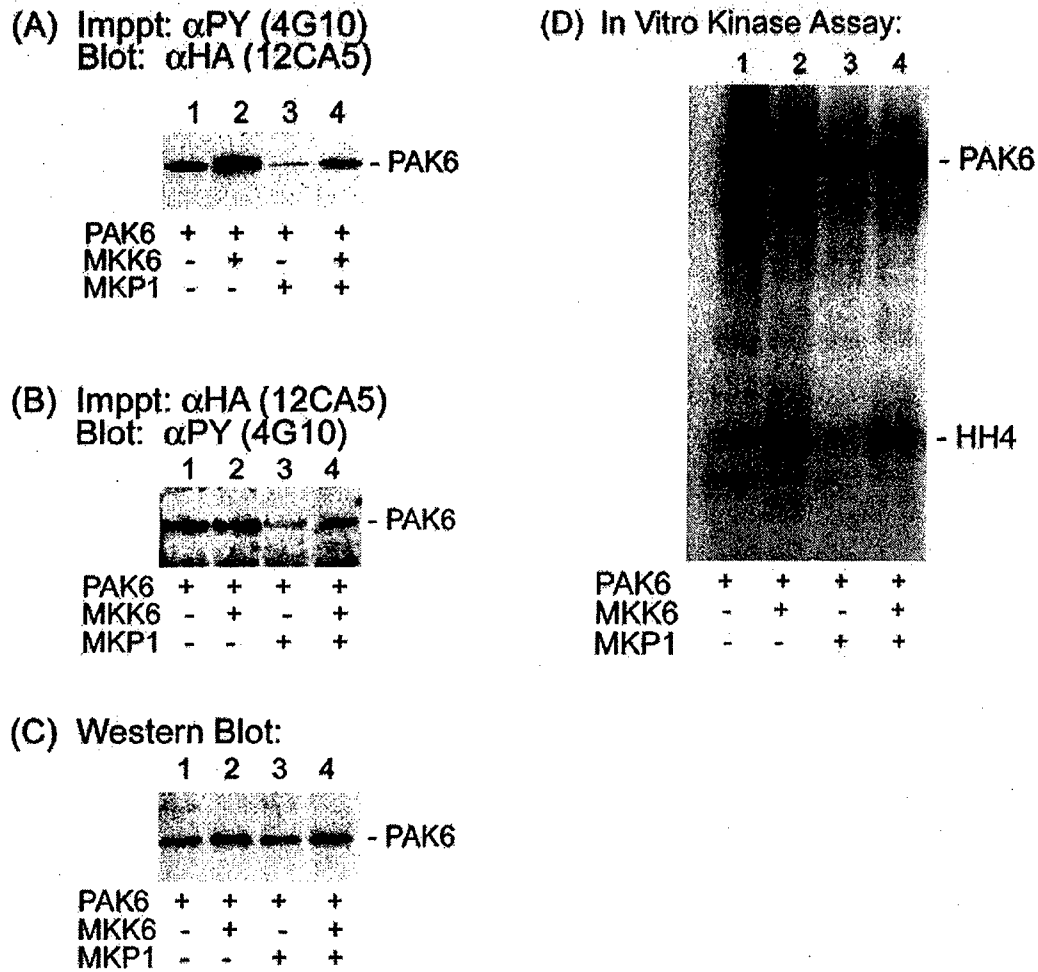


Figure 8: Substitution of Tyr-566 with phenylalanine (Y566F) dampens MKK6-stimulated PAK6 activation

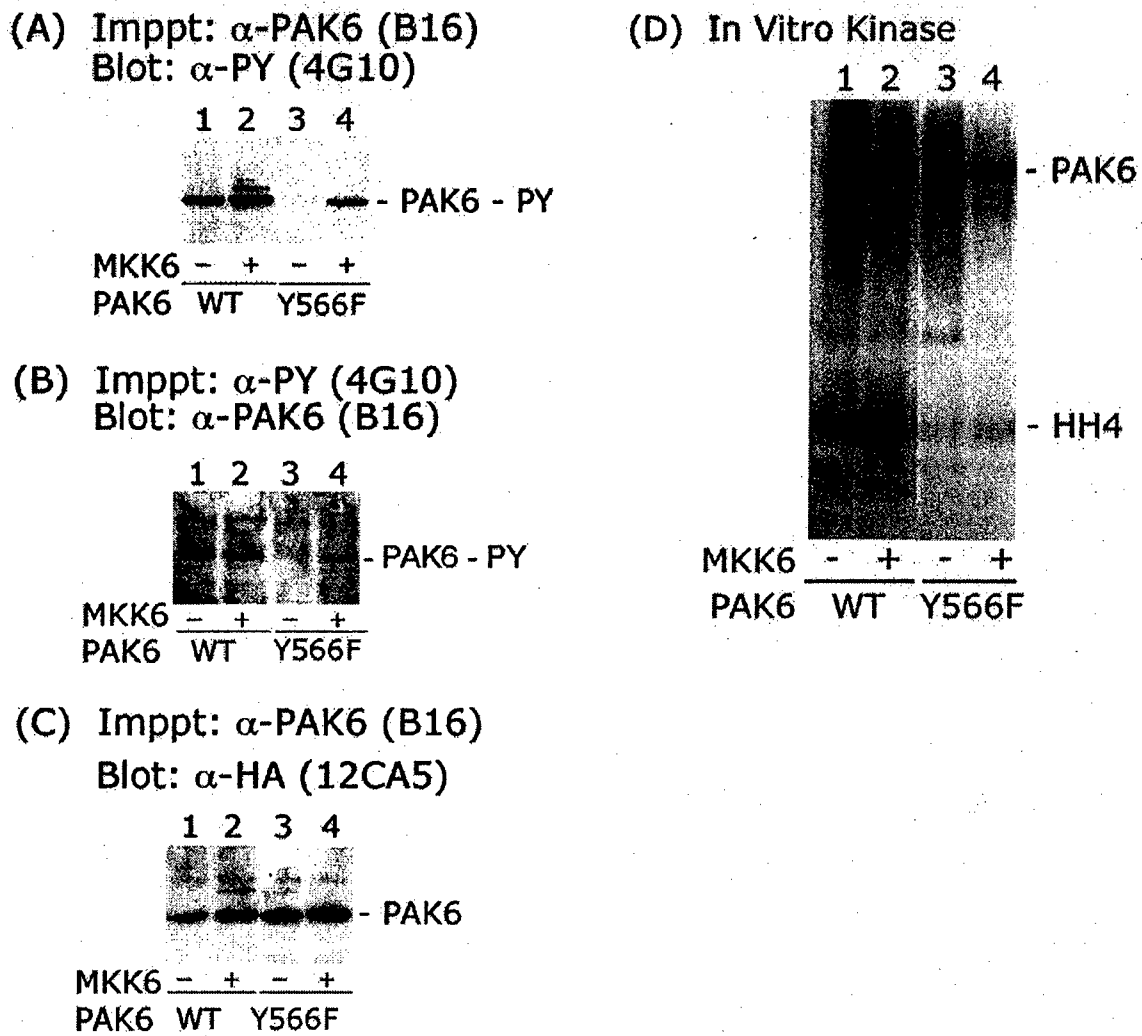
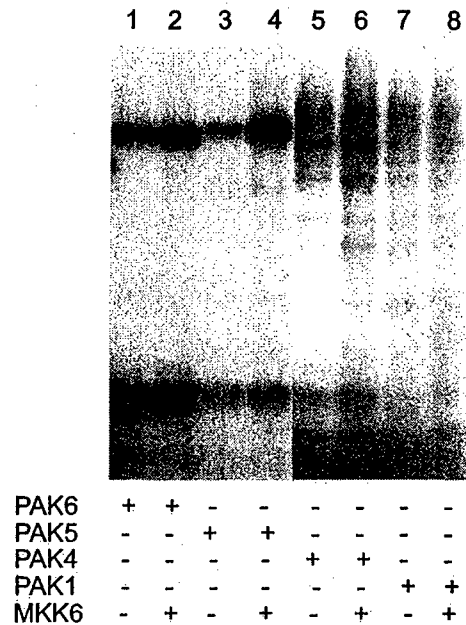


Figure 9: Specificity of MKK6-induced PAK activation

(A) Kinase Activation Domain Alignment:

KINASE	Subdomain	
	VII	VIII
Activation Loop		
PAK6	DFGFCAQISKDVPKRK---	<u>SLVG</u> <u>TPY</u> WMAPE
PAK5	DFGFCAQVSKEVPRRK---	<u>SLVG</u> <u>TPY</u> WMAPE
PAK4	DFGFCAQVSKEVPRRK---	<u>SLVG</u> <u>TPY</u> WMAPE
PAK1	DFGFCAQITPEQSKRS---	<u>TMVG</u> <u>TPY</u> WMAPE
PAK2	DFGFCAQITPEQSKRS---	<u>TMVG</u> <u>TPY</u> WMAPE
PAK3	DFGFCAQITPEQSKRS---	<u>TMVG</u> <u>TPY</u> WMAPE
p38a	DFGLARHTDDEM-----	<u>TGY</u> VATR WYRAPE
p38b	DFGLARQADEEM-----	<u>TGY</u> VATR WYRAPE
JNK1	DFGLARTAGTSFMM-----	<u>TPY</u> VVTRY YRAPE
JNK2	DFGLARTACTNFMM-----	<u>TPY</u> VVTRY YRAPE
Erk1	DFGLARIADPEHDHTGFL	<u>TEY</u> VATR WYRAPE
Erk2	DFGLARVADPDHDHTGFL	<u>TEY</u> VATR WYRAPE

(B) In Vitro Kinase Assay:



(C) Western Blot:

